

Hydrolytic Behavior of Isoalloxazines Related to Riboflavin I

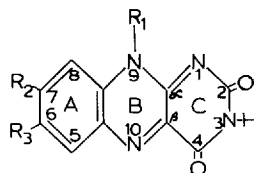
Identification of an Intermediate and Products in the Base-Catalyzed Degradation of 9-Methylisoalloxazine

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Hydrolytic breakdown of 9-methylisoalloxazine was investigated in pH 9.0 borate buffer and in 0.5 *N* sodium hydroxide. Spectral and thin-layer chromatographic analysis of the degraded solutions revealed that different products were formed depending on the reaction conditions. Thus, at high pH and in the presence of air, 1,2-dihydro-1-methyl-2-keto-3-quinoxaline carboxylic acid was predominantly formed, while at pH 9.0, 1,2,3,4-tetrahydro-1-methyl-2,3-dioxo-quinoxaline was the main product. At pH 9.0 under anaerobic conditions the predominant products were the keto acid and 1,2-dihydro-1-methyl-2-oxo-quinoxaline. Examination of the degrading solutions by thin-layer chromatography revealed the presence of an intermediate in the reaction pathways. This intermediate was successfully isolated and identified to be 5-(6-methylaminophenylimino) barbituric acid anil.

THE PHOTOLYTIC instability of riboflavin (I) has been well documented. The intermediates and the reaction products have been isolated and identified, and the mechanistic picture is fairly clearly understood (1-4). This mode of degradation in pharmaceutical systems can, of course, be prevented by use of containers which are opaque to deleterious radiation. Riboflavin and other flavins, however, in aqueous solutions can undergo hydrolytic decomposition involving the isoalloxazine nucleus and which occurs with concomitant loss of biological activity. This latter route of degradation is particularly pronounced in alkaline media, and several studies of both qualitative and quantitative nature have been reported. Among the first investigations were those of Kuhn and Rudy (5). They degraded lumiflavin (II) in alkaline medium and were able to isolate urea and 1,2-dihydro-1,6,7-trimethyl-2-keto-3-quinoxaline carboxylic acid from the reaction mixture. Other reports including those of Surrey and Nachod (6) substantiated these findings. The first detailed kinetic study on the hydrolysis of riboflavin has been reported by Farrer and MacBwan (7). They studied the hydrolysis of riboflavin over the pH range 0.5 to 12.0 and observed the reaction to be general acid-base catalyzed. They also followed the progress of the reaction by paper chromatography and noted transient appearance of lumichrome. Their completely degraded

solutions showed the presence of the quinoxaline carboxylic acid and an unidentified compound with mauve fluorescence under ultraviolet light. The presence of several unidentified compounds in degraded solutions of riboflavin and other isoalloxazines has been also reported by other workers. Thus, Svobodova (8), who resolved solutions of lumiflavin which were partially degraded under alkaline conditions, was able to see several unidentified spots on chromatographic paper strips. Similar studies were also conducted by a group of workers led by Wada (9), who reported as many as seven unidentified spots. These reports together with some preliminary studies on 3,9-dimethylisoalloxazine (10) gave some indication as to the complexity of the hydrolytic breakdown and emphasized the need for further work. This communication summarizes the results of the preliminary studies on this problem.



	R ₁	R ₂	R ₃
Riboflavin (I)	Ribityl	CH ₃	CH ₃
Lumiflavin (II)	CH ₃	CH ₃	CH ₃
9-Methylisoalloxazine (III)	CH ₃	H	H

9-Methylisoalloxazine (III), rather than riboflavin, was chosen for the purpose of detailed investigation because of its stability to light and because the absence of ribityl side chain precluded certain complexities observed with riboflavin (11). Like riboflavin, 9-methylisoalloxazine is susceptible to the base-catalyzed degradation and is a useful model for studies on the properties of iso-

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alloxazines. The hydrolysis of this compound was investigated under conditions of high pH (0.5 *N* NaOH) and at pH 9.0. In addition, the pH 9 systems were also studied under anaerobic conditions. A chromatographic separation technique was developed to follow the progress of the reaction under different conditions. Identification of the reaction intermediates and the products was accomplished by comparison of their R_f values, fluorescence, and spectral characteristics with those of known compounds.

As it will be seen, spectral and thin-layer chromatographic analysis of solutions of 9-methylisalloxazine that were degraded under different experimental conditions revealed that different products were formed depending upon the reaction conditions. Thus, in the aerobic systems maintained at high hydroxide-ion concentrations, 1,2-dihydro-1-methyl-2-keto-3-quinoxaline carboxylic acid (IV) was the major product, while at pH 9 and in the presence of air 1,2,3,4-tetrahydro-1-methyl-2, 3-dioxo-quinoxaline (V) was predominantly formed. At the same pH, in the absence of air, the predominant products were the keto carboxylic acid and 1,2-dihydro-1-methyl-2-oxo-quinoxaline (VIII). Evidence was obtained to indicate that the anil of 5-(6-methylaminophenylimino) barbituric acid (VI) and/or 1,2-dihydro-1-methyl-2-oxo-quinoxaline-3-carboxyureide (VII) was an intermediate in the reaction under all conditions investigated.

EXPERIMENTAL

Materials.—9-Methylisalloxazine was obtained as described previously (12).

5-(6-Methylaminophenylimino) Barbituric Acid Anil (VI).—This was synthesized according to the method of Kuhn (13). The anil was purified by dissolving it in water, by adding base, and precipitating the dissolved compound through acidification. The procedure was repeated several times until the precipitated solid was no longer yellowish. Finally, the compound was crystallized from acetone-water to yield silky white needle-like crystals, m.p. 242–244° dec. The anil was titrated potentiometrically under nitrogen atmosphere in DMF, against standard base, and gave a neutral equivalent of 252. Theoretical, based on the molecular formula $C_{11}H_{10}N_4O_3 \cdot \frac{1}{2}H_2O$ and one titrable hydrogen, was 255.

Anal.—Calcd. for $C_{11}H_{10}N_4O_3 \cdot \frac{1}{2}H_2O$: C, 51.76; H, 4.31; N, 21.96. Found: C, 52.22; H, 4.77; N, 21.70.

The anil was reported by Kuhn (13) and later by Tishler and co-workers (14) to be yellow in color. It appears that their product was contaminated with the ureide (VII) to which the anil can be easily converted by simple heat or acid treatment. Both Kuhn and Tishler used aqueous acetic acid as their crystallizing solvent and as a result probably obtained a mixture of the anil and the ureide.

1,2-Dihydro-1-methyl-2-keto-3-quinoxaline Carboxylic Acid (IV).—This was synthesized from the anil

(VI) in the manner suggested by Kuhn (13). The compound which was obtained as straw-yellow needle-like crystals melted at 176° dec. [King and Clark-Lewis (15) gave m.p. 173–174° dec.] The equivalent weight, determined by nonaqueous titration against methanolic KOH, was 201.6. Theoretical, based on the molecular formula of $C_{10}H_8N_2O_3$ and one titrable hydrogen, was 204.

Anal.—Calcd. for $C_{10}H_8N_2O_3$: C, 58.82; H, 3.92; N, 13.73; O, 23.53. Found: C, 58.97; H, 4.03; N, 13.61; O, 23.53.

1,2-Dihydro-1-methyl-2-oxo-3-quinoxaline Carboxy Ureide (VII).—This was prepared in the manner suggested by King *et al.* (15), m.p. 246–248° (uncorrected). [Lit. m.p. 248° (15).]

1,2,3,4-Tetrahydro-1-methyl-2,3-dioxo-quinoxaline (V).—This was synthesized according to the procedure described by Miles *et al.* (16). A mixture of equimolar quantities of *N*-methyl-*o*-phenylenediamine hydrochloride and anhydrous oxalic acid was heated at 160° in an oil bath for about 15 min. The melt was cooled and crystallized several times from anhydrous methanol to yield shiny white needle-shaped crystals, m.p. 282–283° (uncorrected).

Anal.—Calcd. for $C_9H_8N_2O_2$: C, 61.36; H, 4.55; N, 15.91. Found: C, 61.22; H, 4.7; N, 15.76.

1,2-Dihydro-1-methyl-2-oxo-quinoxaline (VIII).—This was prepared by sublimation of the keto acid (IV) under reduced pressure at 160° (17). The compound was purified through resublimation, m.p. 119–120° (uncorrected).

Anal.—Calcd. for $C_9H_8N_2O$: C, 67.50; H, 5.00; N, 17.50. Found: C, 67.70; H, 5.08; N, 17.24.

All other chemicals were obtained from commercial sources. The microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

Spectral Studies.—Solutions of 9-methylisalloxazine and of the anil were completely degraded in pH 9.0 borate buffer and in 0.5 *N* sodium hydroxide. The spectra of the degraded solutions were obtained on a Beckman DB spectrophotometer.

Thin-Layer Chromatographic Studies.—The progress of the hydrolysis of 9-methylisalloxazine in pH 9.0 borate buffer and in 0.5 *N* base was followed by thin-layer chromatography. Silica Gel G thin-layer plates (4 × 20 cm.²) of 500 μ thickness were used. Sixty-microliter samples were withdrawn at different time intervals and spotted on the plates. Care was exerted to limit the diameter of the spots to about 5 mm. The chromatograms were developed using $CHCl_3$ -acetic acid-pyridine (5:1:1). The solvent front was allowed to run 10 cm. from the point of application of samples. The developed plates were warmed to remove the solvent and examined under long-wave ultraviolet light. The different fluorescent spots were identified by comparison of their R_f values with those of authentic samples.

Isolation and Characterization of the Anil (VI) from Partially Degraded Reaction Mixture.—9-Methylisalloxazine was degraded in 0.5 *N* sodium hydroxide at 45° for 1.5 hr. This partially degraded solution was rapidly cooled and quickly acidified to pH 5.0. The acidified solution was exhaustively extracted with ether. The ethereal solution was dried over anhydrous sodium sulfate, filtered, and the solvent removed under vacuum. The residue was dissolved in 2 ml. of absolute

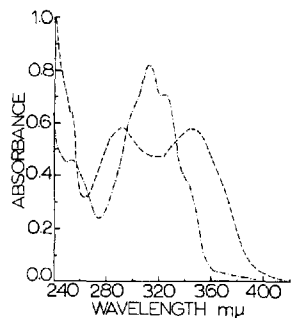


Fig. 1.—Spectra of the solutions of 9-methylisoalloxazine that were degraded in pH 9.0 borate buffer (---) and in 0.5 *N* sodium hydroxide (.....).

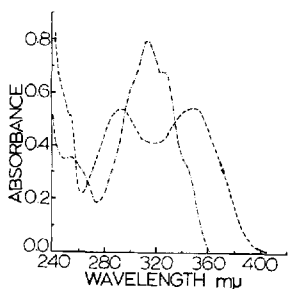


Fig. 2.—Spectrum of the keto acid in 0.5 *N* sodium hydroxide (.....) and that of the dioxo compound in pH 9.0 borate buffer (---).

ethanol and introduced over a silica gel column (10 cm. long and 1 cm. in diameter). Elution was effected with anhydrous ether and the eluate collected in 5-ml. fractions. Earlier studies with synthetic mixtures of the anil (VI), the ureide (VII), and the isoalloxazine (III) showed that such a procedure was effective in separating the anil from the other two compounds. The anil was contained in the first few fractions of eluate.

Anaerobic Studies.—Here the reaction systems containing 9-methylisoalloxazine in pH 9.0 borate buffer were formulated in 10-ml. ampuls. Each ampul was thoroughly flushed with nitrogen and sealed immediately. The sealed ampuls were suspended in a constant-temperature bath adjusted at 45°. The ampuls were opened at different times and the spectrum of each solution was obtained. Concomitant with this, 60- μ l. samples were developed on Silica Gel G thin-layer plates using CHCl_3 -acetic acid-pyridine (5:1:1) solvent system. The developed plates after drying were examined under long-wave ultraviolet light, and the R_f values of different spots were compared with those obtained with known compounds.

RESULTS

Isolation and Identification of Reaction Products.

A preliminary indication of the dependence of the reaction pathways on the hydroxide ion concentrations was obtained by comparison of the spectra of solutions of 9-methylisoalloxazine which were degraded under two different conditions of hydroxide ion concentration. Figure 1 illustrates such spectra. The striking difference between the spectra clearly indicated that different products were favored depending on the pH of the reaction medium. In Fig. 2 are illustrated the spectra of two possible decomposition products, *i.e.*, the keto acid and the dioxo

compound. It can be seen from Figs. 1 and 2 that the spectrum of the isoalloxazine solution degraded at pH 9.0 closely resembled that of the dioxo compound while that of the solution degraded in 0.5 *N* sodium hydroxide corresponded to that of the keto acid. The results of the thin-layer chromatographic studies (see t_{∞} samples) summarized in Figs. 3 and 4 revealed that both the keto acid and the dioxo compound were formed under all conditions of pH. However, the relative intensities of the spots corresponding to the two compounds, together with spectral data, demonstrated that the keto acid was the major product at high pH, while at the lower pH, the dioxo compound was mainly formed.

It should be pointed out that the appearance of the dioxo compound as a reaction product was unexpected since the formation of this compound from the parent isoalloxazine must involve both hydrolytic and oxidative reactions. It was the work of Miles *et al.* (16) which presented fluorescence, spectral, and chromatographic properties of a similar compound formed in the bacterial degradation of riboflavin that first drew the authors' attention to the possibility that the dioxo compound was indeed formed.

Thin-Layer Chromatographic Studies.—Fluorescence characteristics under long-wave U.V. light and R_f values for various compounds which could conceivably be formed in the reaction are presented in Table I. It can be seen here and in Fig. 3, which illustrates the progress of the reaction in 0.5 *N* base, that a compound with sky-blue fluorescence and with an R_f approximately 0.38, appeared transiently during the hydrolysis. This R_f value and the fluorescence characteristics agreed with that of a suspected intermediate, *viz.*, the anil compound. The results of a similar study at pH 9.0 are presented in Fig. 4. Only three spots are apparent here, those of the parent compound, the dioxo compound, and a very weak one due to the keto acid.

Isolation and Characterization of the Intermediate from the Reaction Mixture.—A suspected intermediate, the anil, was successfully isolated from partially degraded solution of the isoalloxazine.



Fig. 3.—Chromatograms illustrating the progress of hydrolysis of 9-methylisoalloxazine in 0.5 *N* sodium hydroxide and at 45°. Key: A, keto acid; B, anil; C, isoalloxazine; D, dioxo compound.

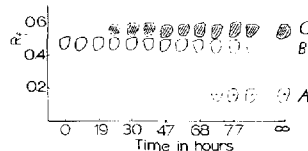


Fig. 4.—Chromatograms illustrating the progress of hydrolysis of 9-methylisoalloxazine in pH 9.0 borate buffer and at 45°. Key: A, keto acid; B, isoalloxazine; C, dioxo compound.

The isolation was achieved through ether extraction of the acidified reaction mixture. Further purification of the isolated material was accomplished through the use of silica gel columns. The spectrum of the isolated compound is shown in Fig. 5 and is identical with that of the pure anil. In addition, the isolated compound exhibited the same fluorescence and chromatographic behaviors as authentic anil. Figure 5 also shows the spectrum of the ureide which could also conceivably be an intermediate in the reaction pathway. It was observed, however, that the ureide in aqueous solution was rapidly and almost quantitatively converted to the anil to form what appeared to be an equilibrium mixture. A typical spectrum of such a mixture in 0.01 *N* HCl is also shown in the figure. The equilibrium was found to be pH dependent, and the anil appeared to be the predominant species in the neutral and alkaline pH range.

Solutions of the anil were formulated in 0.5 *N* NaOH and in pH 9.0 borate buffer and the compound was allowed to degrade. Spectra of the degraded solutions are shown in Fig. 6. These spectra are remarkably similar to those obtained with the parent isoalloxazine.

TABLE I.— R_f VALUES AND THE FLUORESCENCE CHARACTERISTICS OF VARIOUS COMPOUNDS ON SILICA GEL G THIN-LAYER PLATES IN CHCl_3 -ACETIC ACID-PYRIDINE (5:1:1) SOLVENT SYSTEM

Compd.	Fluorescence Behavior under U.V.	R_f Values
Isoalloxazine (III)	Yellow-green	0.45
Keto acid (IV)	Sky-blue	0.18
Dioxo compd. (V)	Dark blue	0.57
Anil (VI)	Faint sky-blue turning greenish	0.38
Ureide (VII)	Yellow-green	0.78
Quinoxaline compd. (VIII)	Dark blue	0.89
Unidentified compd.	Aquamarine	0.69

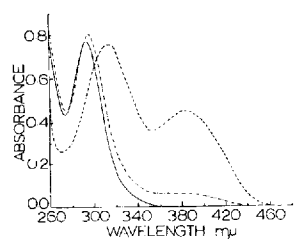


Fig. 5.—Spectra of the anil (—), the ureide (---), and that of the equilibrium mixture in 0.01 *N* HCl (-·-·-).

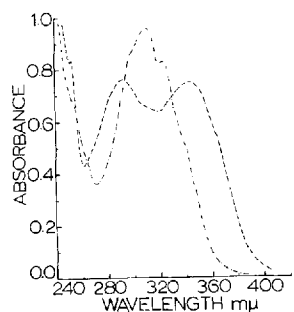


Fig. 6.—Spectra of the solutions of the anil that were degraded in 0.5 *N* sodium hydroxide (---) and in pH 9.0 borate buffer (-·-·-).

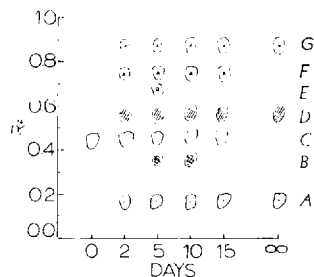
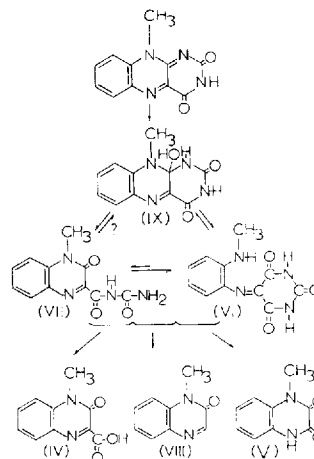


Fig. 7.—Chromatograms illustrating the progress of the anaerobic hydrolysis of 9-methylisoalloxazine in pH 9.0 borate buffer and at 45°. Key: A, keto acid, B, anil; C, isoalloxazine; D, dioxo compound; E, unidentified compound; F, ureide; G, quinoxaline compound.

Anaerobic Studies.—Thin-layer chromatographic results of anaerobic hydrolysis of the isoalloxazine at pH 9.0 are presented in Fig. 7. It can be seen here that the degrading solution of the isoalloxazine under these conditions exhibited spots which corresponded to the keto acid and the quinoxaline compound. In addition, it also showed the anil, the ureide, and an unidentified spot exhibiting aquamarine fluorescence under U.V. light and with an R_f of 0.69.

DISCUSSION

The information which has been collected in this study demonstrated that 9-methylisoalloxazine, in aqueous solution, was transformed into a number of different products, and that the anil compound was an intermediate in such transformations. A proposed scheme for the degradation is presented in Scheme I. The isoalloxazine is seen to be initially



Over-All Reaction Mechanism for the Degradation of 9-Methylisoalloxazine. Scheme I

converted to the anil compound. Such a transformation can possibly result from hydroxide-ion attack at the α carbon to form a carbinolamine (IX), which in turn can rupture to form either the anil (VI) or the ureide (VII). Evidence, which will be presented in a future communication, has been ob-

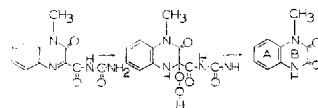
tained to indicate the involvement of such a carbinolamine intermediate. Although the anil compound has definitely been implicated in the reaction scheme, the existence of the ureide is somewhat speculative. As was indicated, however, there is evidence that ureide and anil existed in equilibrium in solution and that the equilibrium favored the anil under the pH conditions which prevailed in this study.

The interesting and somewhat unexpected observation that high pH degradation yielded the keto acid (IV) as the predominant product, while at pH 9.0 the dioxo compound (V) was the main product, can be explained by noting that the anil compound could undergo further reaction by both hydrolytic and oxidative routes and that the relative concentrations of the products would depend on the relative rates of the processes. Thus, at high pH the hydrolytic reaction was much faster than that of oxidation, while at pH 9 the reverse was true. Exclusion of air from the systems at pH 9 markedly decreased the rate of oxidation and made apparent that the hydrolytic reaction which yielded the keto acid was also operant under these conditions. Another reaction pathway which resulted in the formation of the quinoxaline compound (VIII) also became apparent under anaerobic conditions.

The failure to detect the anil in solutions of the isoalloxazine which were degraded at pH 9 in the presence of air can also be explained on the basis of relative reaction rates. It appears that under these conditions, the rate of disappearance of the anil was faster than the rate of its formation from the parent compound. Support of this stems from the fact that solutions of isoalloxazine, which were partially degraded at pH 9 in the absence of air, where the oxidative transformation of the intermediate anil was suppressed, did contain demonstrable concentrations of anil. That the anil was formed as an intermediate under all conditions investigated is also indicated by the spectral studies on completely degraded solutions, which showed that under both conditions of pH the anil yielded the same reaction products as the parent isoalloxazine.

The anaerobic studies with the isoalloxazine also revealed the formation of yet another compound with a weak aquamarine fluorescence under U.V. light and with an R_f value of 0.69 in the solvent system used. This compound has, as yet, not been identified.

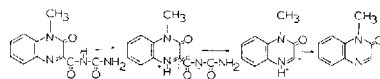
Although no definitive evidence was obtained to indicate whether the formation of the final products proceeded through the anil or the ureide, the presence of the intact B ring in the products suggest the latter as the intermediate. Simple hydrolysis would yield the keto acid and urea. It can be speculated that the dioxo compound was formed through a hydroperoxide intermediate such as that depicted in Scheme II, which could undergo homolytic cleavage as shown. Studies on solutions of the keto acid showed that it was stable under the experimental conditions employed and did not undergo any detectable degree of decarboxylation even after prolonged exposure to the reaction conditions. Thus, the quinoxaline compound (VIII) was not derived



A Possible Mechanism for the Formation of Dioxo Compound from the Ureide.

Scheme II

from the keto acid (IV). It could conceivably arise directly from the ureide (VII) which has a weakly acidic imino hydrogen and could be considered to be analogous to a quinaldic carboxylic acid. Such acids are known to decarboxylate readily particularly when unionized (18). A possible mechanism for such a transformation of the ureide is given in Scheme III.



A Possible Mechanism for the Formation of the Quinoxaline Compound from the Ureide.

Scheme III

The formation of an oxidation product analogous to the dioxo compound which was identified in this study, was reported by Stadtman and co-workers (16, 19) to result from bacterial degradation of riboflavin and to involve both hydrolytic and oxidative enzymatic mechanisms. This apparent analogy between chemical and biological systems is interesting and suggests that information on the hydrolytic behavior of isoalloxazines in simple chemical systems might be of significance in gaining a better understanding of certain biochemical reactions involving riboflavin.

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